

OLLE HEBY

THE POLYAMINES

THEIR INTERACTIONS WITH NUCLEIC ACIDS

AND SOME ASPECTS OF THEIR ROLE

IN GROWTH AND DEVELOPMENT

LUND 1971

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IN GROWTH AND DEVELOPMENT

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The present thesis is based upon the following papers:

1. Agrell I, Heby O: Interactions of polyamines and acid macromolecules observed in double diffusion in gel experiments. Exp Cell Res 50:668-671, 1968
2. Heby O, Agrell I: Observations on the affinity between polyamines and nucleic acids. Hoppe Seyler's Z Physiol Chem 352:29-38, 1971
3. Agrell I, Heby O: Interaction between spermine and histones at nucleoprotein formation. Hoppe Seyler's Z Physiol Chem 352:39-42, 1971
4. Heby O: Polyamine variations during postembryonic development of the blowfly *Calliphora erythrocephala*. Insect Biochem (in press)
5. Holm B, Heby O: Putrescine and spermidine changes in heat-synchronized *Tetrahymena* populations. Z Naturforsch 26b: 604-606, 1971
6. Heby O, Lewan L: Putrescine and polyamines in relation to nucleic acids in mouse liver after partial hepatectomy. Virchows Arch Abt B Zellpath 8:58-66, 1971
7. Andersson G, Heby O: Polyamine and nucleic acid concentrations in Ehrlich ascites carcinoma cells and liver of tumor-bearing mice at various stages of tumor growth. J Nat Cancer Inst (in press)

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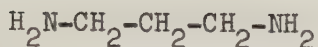
CONTENTS

INTRODUCTION	4
PART I. POLYAMINE-NUCLEIC ACID INTERACTIONS	8
A. The affinity of polyamines for nucleic acids.....	9
1. Qualitative analyses	9
2. Quantitative analyses	10
a. Native DNA	11
b. RNA	12
c. sRNA	13
d. Denatured DNA	14
B. The effect of polyamines on nucleohistone formation	15
1. Nucleohistone formation	16
2. Interference of polyamines with nucleohistone formation ...	19
3. Physical state of the chromatin	20
C. The role of polyamines and histones in gene expression	22
PART II. POLYAMINES IN GROWTH AND DEVELOPMENT	24
A. Embryonic and postembryonic development	25
B. Cell growth and synchronous division	29
C. Regenerative growth of the liver	31
D. Tumour growth	34
1. Studies on the tumour	34
2. Studies on the liver of tumour-bearing animals	35
SUMMARY AND CONCLUSIONS	37
ACKNOWLEDGEMENTS.....	39
REFERENCES	40

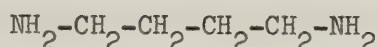
*aliquantulus majoris, ut fig. A. Praeterea, alio modo in
palliditate, ut in crystallinis presentibus. These crystals were
presumably spermine phosphate. In the subsequent 30 years*

INTRODUCTION

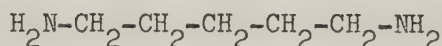
Naturally occurring polyamines include the following compounds:



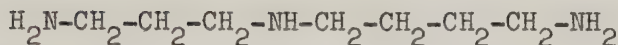
1,3-diaminopropane



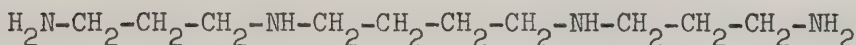
1,4-diaminobutane; putrescine



1,5-diaminopentane; cadaverine



N-(3-aminopropyl)-1,4-diaminobutane; spermidine



N,N'-bis-(3-aminopropyl)-1,4-diaminobutane; spermine

The earliest mention of polyamines is to be found in Antoni van Leeuwenhoek's famous communication to the Royal Society of London, dated November 1677, in which he first described spermatozoa. In this letter he also reported the discovery of a crystalline substance in human semen: "*Et cum praedicta materia paucillum temporis steterat, in ea observabantur trilaterales figurae ab utraque parte in aculeum desinentes, quibusdam longitudo minutissimae arenae, aliquae aliquantulum majores, ut fig. A. Praeterea, adeo nitidae ac pellucidae, ac si crystallinae fuissent*". These crystals were presumably spermine phosphate. In the subsequent 300 years

this substance was rediscovered by several investigators. The other amines have had a considerably shorter history than spermine.

Putrescine and cadaverine were first discovered in decomposing animal material, cadavers, and in the urine of cystinuric patients.

The biosynthesis of polyamines was first studied in bacteria (Tabor, Rosenthal & Tabor 1956, 1957, 1958, Tabor & Tabor 1964) but there is evidence that similar pathways are present in animals (Tabor, Rosenthal & Tabor 1956, Raina 1962, 1963, Jänne & Raina 1966, Jänne 1967, Raina & Jänne 1968a, Pegg & Williams-Ashman 1968a, 1968b, 1969, 1970, Pegg 1970, Pegg, Lockwood & Williams-Ashman 1970, Raina & Hannonen 1970, 1971). An outline of the metabolic pathways leading to the biosynthesis of polyamines, and of the interrelationships between polyamines and other metabolites is given in Fig. 1.

In recent years several observations have heightened interest in the polyamines. Studies on the role of these compounds as growth factors for various microorganisms (Herbst & Snell 1949, Guirard & Snell 1964, Inouye & Pardee 1970) and a mammalian cell line (Ham 1964) indicated that they might be of widespread biological significance. This hypothesis was supported by analytical studies, which showed the occurrence of polyamines in animals, bacteria, plants, and viruses (Tabor & Tabor 1964). A possible functional connection with the nucleic acids was suggested by the *in vitro* affinity of putrescine, spermidine, and spermine for these molecules.

Further details of the biosynthesis and occurrence of polyamines, and a survey of their possible functions, can be found in a review by Tabor & Tabor (1964), which covers most of the litera-

ture on polyamines prior to 1964. Subsequent investigations have been reviewed by Bachrach (1970) and Stevens (1970).

Although there is yet no unifying hypothesis to correlate all the experimental findings, there is no longer any doubt about the potential biological significance of the polyamines. In order better to understand how polyamines act in the living cell it seemed appropriate to ascertain the polyamine-nucleic acid interaction in vitro, and to determine the exact relationships between the concentrations of polyamines and nucleic acids in various growth systems.

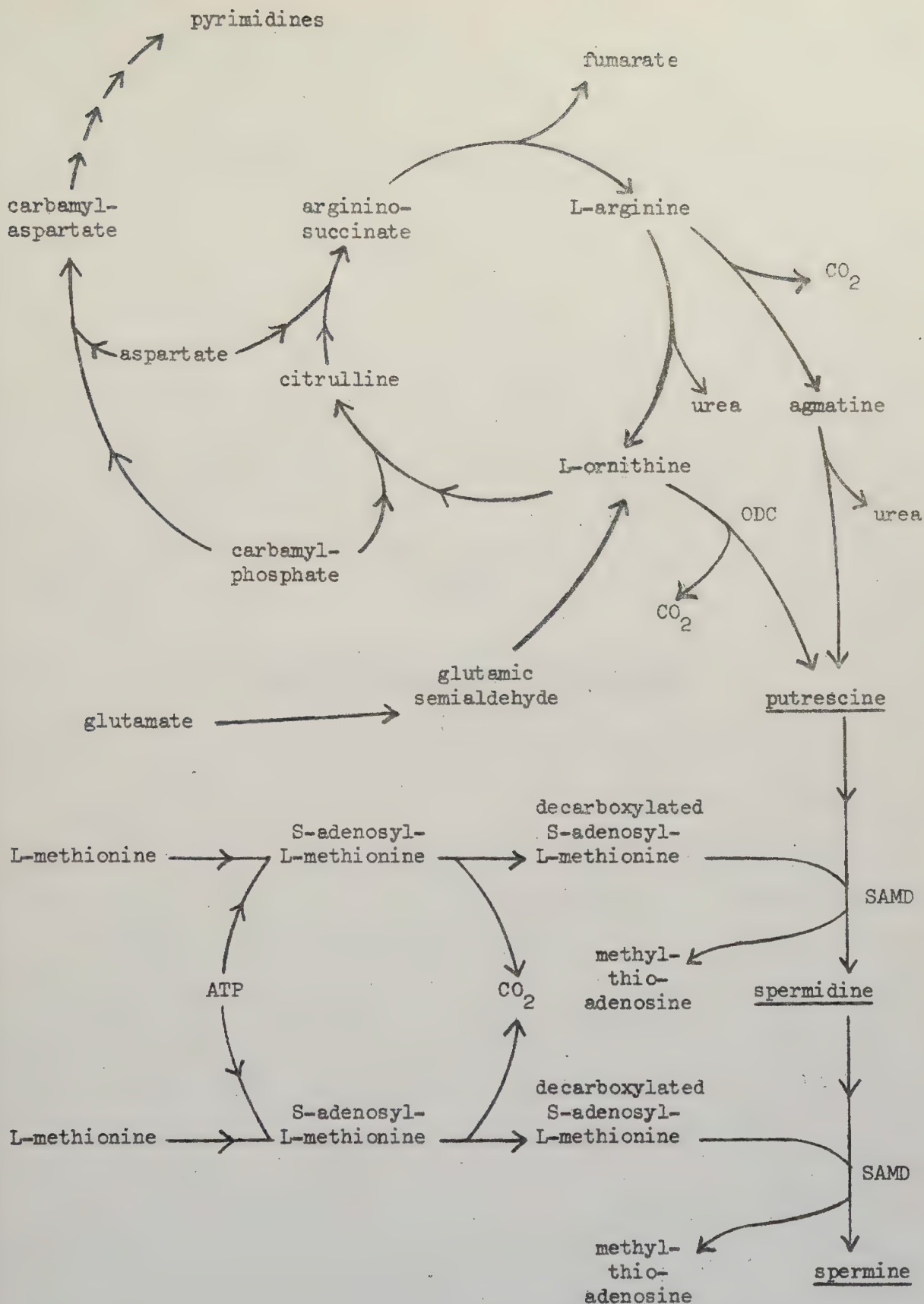


Fig. 1. Pathways involved in the biosynthesis of polyamines, and showing their interrelationships with other metabolites. The route to putrescine, from arginine via agmatine, has only been demonstrated in bacteria (Morris & Pardee 1966). ATP = adenosine triphosphate, ODC = ornithine decarboxylase, SAMD = S-adenosyl-L-methionine decarboxylase.

PART IPOLYAMINE-NUCLEIC ACID INTERACTIONS

A. The affinity of polyamines for nucleic acids (Papers 1 and 2).

Previous findings suggest that the effects of spermidine and spermine on various cellular and subcellular systems are largely attributable to interaction between these amines and the nucleic acids (Keister 1958, Razin & Rozansky 1959, Ochoa & Weinstein 1965, Raina & Telaranta 1967, Raina & Jänne 1968b). Better understanding of the nature and extent of this interaction might help to elucidate the biological functions of polyamines. Spermidine and spermine, by virtue of their highly cationic nature (all the imino and amino groups are protonated at physiological pH values), can complex with DNA and RNA molecules, apparently by neutralization of the negatively charged phosphate groups of the nucleic acids (Tabor 1962, Felsenfeld 1962). This interaction serves to stabilize the nucleic acids, as is manifested by an increased denaturation temperature (T_m) (Tabor 1962, Mandel 1962, Mitra & Kaesberg 1963, Goldstein 1966, Hirschman, Leng & Felsenfeld 1967), an increased resistance to breakage by hydrodynamic shear (Kaiser, Tabor & Tabor 1963) and an inhibition of enzymic degradation (Herbst & Doctor 1959, Bachrach & Eilon 1969). At sufficiently high amine concentrations, the complexes with the nucleic acids form precipitates (Razin & Rozansky 1959).

1. Qualitative analyses (Paper 1).

In a pilot experiment, we made use of the finding that polyamine-nucleic acid complexes can form precipitates. The interaction between various polyamines and nucleic acids was observed

by precipitation in double diffusion experiments in agarose gels. Spermidine and spermine gave precipitation lines with various types of DNA and RNA, whereas the diamines, putrescine and cadaverine, did not. Spermine was more active than spermidine in precipitating the various nucleic acids, and the DNA's were more easily precipitated than the RNA's. We also observed a competition between the polyamines and the diamines for sites on the nucleic acids. As predicted, nucleic acid-spermidine precipitation was easier to prevent than nucleic acid-spermine precipitation. Furthermore, RNA precipitation was more easily inhibited than was that of DNA. Inorganic cations are also known to interfere with the formation of polyamine-nucleic acid precipitates (Razin & Rozansky 1959).

These observations and many others can be explained by considering the polyamines as polyvalent cations; indeed some of the effects of the polyamines are comparable with those obtained with Ca^{2+} or Mg^{2+} . The polyamines, however, owing to their polybasic nature, have a particularly high affinity for the cellular polyanions, and thus exert more pronounced effects at lower concentrations (Doctor, Fournier & Thornsvarð 1970, Khawaja & Raina 1970, Tanner 1967). Although all these observations were made in vitro, it seems likely that the stabilizing properties of the polyamines constitute an important aspect of their physiological function.

2. Quantitative analyses (Paper 2).

Molecular models based on X-ray studies (Liquori, Costantino, Crescenzi, Elia, Giglio, Puliti, De Santis Savino & Vitagliano 1967, Suwalsky, Traub, Shmueli & Subirana 1969) indicate that all the basic

groups of a polyamine molecule are capable of forming electrostatic bonds with phosphate groups on the native DNA molecule. Since no quantitative analyses of polyamine-nucleic acid complexes had appeared to confirm this, we undertook a study of the relationships between various nucleic acids and polyamines in precipitates. In particular, we sought to investigate the mode of attachment between polyamines and RNA.

a) Native DNA.- Equimolar amounts of negatively charged phosphate groups and protonated nitrogen groups were found in the DNA-spermine precipitates, which seemed to denote an ideal molecular arrangement between the constituents, consistent with the model proposed by Liquori et al. (1967) and Suwalsky et al. (1969). Thus, in the perfect, double helical, and complementary conformation of DNA, the spermine molecules might occupy the small groove, neutralizing all the phosphate groups of the molecule and binding its two strands together. Moreover, the ability of spermine to precipitate DNA suggests that there may also be intermolecular spermine bridges, cross-linking the DNA molecules.

The DNA-spermidine precipitates showed a base deficit, possibly due to competition between the spermidine molecules for stereospecific sites in the small groove of the DNA molecule. Thus the model proposed by Liquori et al. (1967) for the DNA-spermidine complex, which assumes an equimolar interaction between DNA and spermidine, cannot be valid. An alternative model was therefore proposed. If intermolecular spermidine bridges occur at all, they must be much weaker than spermine bridges. In salt-free solutions,

however, such bridges may contribute to precipitability. The dependence of precipitation upon intermolecular bridges may explain why no DNA-spermidine precipitates were formed in 0.12 M NaCl, whereas the DNA-spermine precipitates were almost unaffected by this salt.

Since the structure of native DNA is such that the heterocyclic bases are not readily available for combination with polyamines, it can be assumed that the principal role in the interaction with polyamines is played by the phosphate groups and not by the heterocyclic bases.

b) RNA. - Excess base was found in the RNA-spermine precipitates. The conformation of RNA and binding of polyamines to the heterocyclic bases was supposed to contribute to and stabilize the excess base. Precipitation may indicate aggregation due to cross-linkage of the RNA molecules by spermine bridges. Neutralization of the negative phosphate groups by spermine would, however, promote aggregation even without bridge formation, since it would reduce the electrostatic interchain phosphate repulsions. In addition, spermine molecules may exert structural effects within a single RNA molecule. Thus Mitra & Kaesberg (1963) have found that the polyamines are remarkably efficient in preventing the unfolding of turnip yellow mosaic virus RNA and in establishing a compact tertiary structure. By virtue of their strong affinity for nucleic acids, the polyamines may help to establish and maintain the exceedingly compact structures of DNA and RNA found in small viruses. This hypothesis is further supported by the observation

that bacteriophage T_4 contains putrescine and spermidine in amounts sufficient to neutralize about half of the viral DNA (Ames & Dubin 1960). These amines are injected into the bacteria along with the viral DNA (Hershey 1957).

c) sRNA. - The composition of the precipitates formed between sRNA and spermine indicated that some ordered structures of sRNA might be responsible for complex formation. Accordingly, a more regular double-stranded pattern was suggested for sRNA than for more highly molecular RNA. In contrast to the observation by Cantoni (1960), we found no effect of pH within the range tested. The spermine:sRNA ratio was constant along the reciprocal concentration gradient from 0.2:0.8 to 0.9:0.1, in spite of the concomitant pH-gradient (<6.9-4.8). Cantoni (1960) observed that spermine-sRNA complexes were completely soluble at pH 7, completely insoluble at pH 4, and partially soluble at intermediate pH. The discrepancy may be explained by the fact that his experiments were performed at higher ionic strength and with a different sRNA. On the other hand, his observations that 70 % of the sRNA was precipitated with an excess of spermine at pH 5.6 and low ionic strength, and that spermine combined stoichiometrically with sRNA at all H^+ concentrations tested, is in accordance with our results. Using a gel filtration method, Ikemura (1969) found that spermine formed stable complexes with polynucleotides having the antiparallel double helix conformation but not with those having a nonrigid conformation. Furthermore, in accordance with our observation, he reported that more spermine was bound to tRNA than to rRNA and concluded that the stable organized

structure was likely to be responsible for the strong binding with spermine.

2) Denatured DNA. - The action of polyamines on denatured DNA indicated that the polyamines might play a role in rejoining the two strands of the DNA molecule. Conductometric titrations (Huang & Felsenfeld 1970, Felsenfeld & Huang 1971) have also shown that spermine combines with denatured DNA with a 1:1 stoichiometry throughout the range between pH 4 and pH 7. In addition it has been found that protons are displaced from denatured DNA and RNA but not from native DNA when spermine is added (Huang & Felsenfeld 1970, Felsenfeld & Huang 1971). Thus the affinity of spermine is sufficiently great to displace protons from the heterocyclic bases when these are available, as in denatured DNA and various RNA's. It must be stressed, however, that the charge on the phosphate groups is likely to be the dominant factor in the interaction. It can also be suggested that polyamines are also capable of engaging the additional binding sites provided by the heterocyclic bases of denatured DNA (Agarall 1971).

B. The effect of polyamines on nucleohistone formation (Papers 1-3).

If histones are, as seems probable, the natural gene repressors, there must be processes which counteract them under circumstances in which gene activity is required. Several possible "derepressing agents" have been mentioned by various authors.

A possible interference of polyamines with whole histone, extracted from calf thymus deoxyribonucleoprotein with dilute hydrochloric acid (Phillips & Johns 1959), was observed in double diffusion in gel experiments (Paper 1). This observation provoked a more detailed investigation of the possible effect of polyamines on nucleohistone formation.

Since histone is not a homogeneous protein, but a family of closely related proteins, we extended the experiments to include histones fractionated according to method 1 of Johns (1964a). Accordingly, 5 main fractions were isolated, each accounting for about 20 % of the total histone. The histones are quite small molecules (M.W.: 12,000-22,000), consisting to about 25 % of basic amino acids. They occur in quantities equivalent to the DNA and are probably present in all eukaryotic somatic cell nuclei, regardless of the different functions of the cells. Originally thought to be of high heterogeneity, they are at present considered to exist in not more than 10 and principally as 5 distinct types (F1, F2A1, F2A2, F2B, F3).

F1 and F3, both of which have special characteristics, were used in our experiments. F1 is lysine-rich and has twice as much proline as any other fraction, which is important for the secondary structure of these proteins, since proline tends to inhibit the formation of an α -helix. Accordingly, F1 is the fraction with the lowest

α -helix content (Bradbury, Crane-Robinson, Phillips, Johns & Murray 1965, Bradbury, Crane-Robinson, Goldman, Rattle & Stephens 1967). F3 is arginine-rich, is the only fraction to contain cysteine, and has an alanine N-terminal group. As a whole, the histones are only exceeded by the protamines in their arginine content and basicity, and F1 is unique among proteins in having over 25 % lysine.

1. Nucleohistone formation (Paper 2).

There was a marked difference in the manner in which the two histones combined with the DNA (Paper 2). In agreement with other workers (Johns & Butler 1964, Chipperfield 1967, Agrell 1969, and Ansevin & Brown 1971), we found that less F1 was required to precipitate the DNA than F3 (Fig. 2). F1 is also known to be more effective than F3 in stabilizing DNA against thermal denaturation, and in yielding those nucleohistones which least actively support RNA synthesis (Huang, Bonner & Murray 1964, Johns & Hoare 1970).

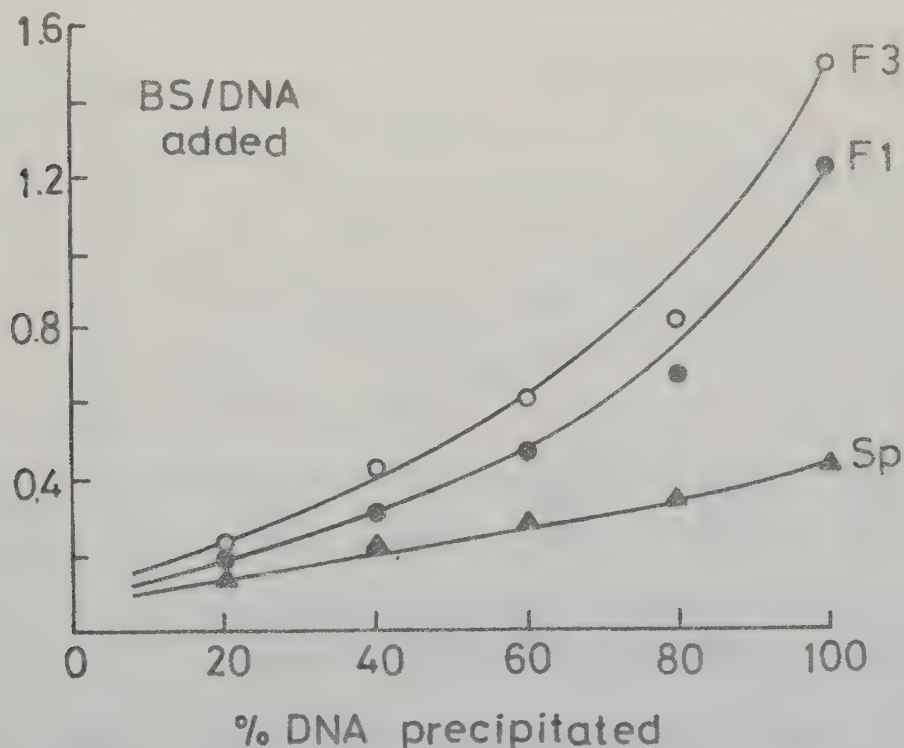


Fig. 2. Efficiency of various basic substances (BS) in precipitating DNA in 0.12 M NaCl-acetate buffer, pH 5. The values are extrapolated from Paper 2, Fig. 2a, taking the initial BS/DNA ratio where 20%, 40%, 60%, 80% and 100% of the DNA was precipitated.

These differences could be accounted for by the type of selective interaction one might expect between a polyanion and polycations varying in charge, and F1 has a higher content of basic amino acids and fewer acidic amino acids than F3.

Contrary to expectation, F1 has the least affinity for DNA. It is the last fraction to be taken up from a mixture of histones (Johns & Butler 1964) and much less F1 is found in the DNA/F1 precipitate than F3 in the DNA/F3 precipitate (Fig. 3) (Johns & Butler 1964, Akinrimisi, Bonner & Ts'o 1965, Agrell 1969, Ansevin & Brown 1971).

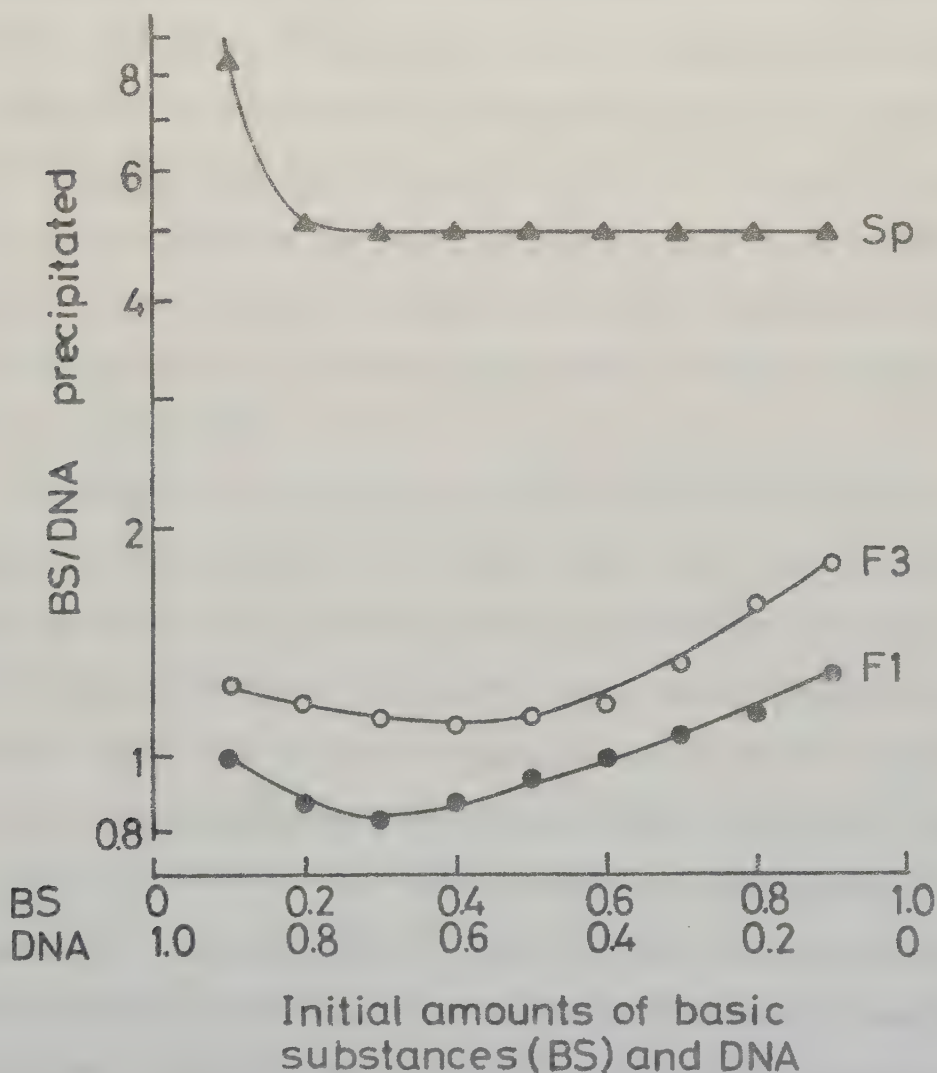


Fig. 3. Affinity of various basic substances (BS) for DNA in 0.12 M NaCl-acetate buffer, pH 5. The values are extrapolated from Paper 2, Figs. 1g and 3a.

It is unlikely that these differences can be accounted for simply by divergent net positive charges on the histone molecules. The ability to satisfy and precipitate DNA is more likely to be proportional to the number of basic amino acids sterically available for combination with the phosphate groups of the DNA.

The finding that smaller amounts of F1 than of F3 are required to precipitate DNA (Fig. 2) also suggests that F1 tends to form cross-links between DNA molecules, whereas F3 tends to align itself along separate DNA molecules (Sluyser & Snellen-Jurgens 1970). The ability of F1 to form cross-links between nucleic acid molecules to a relatively high degree, may be explained by its extended chain conformation. F3 molecules probably have a more compact structure. It has been suggested that the lysine-rich histones function as cross-links between different nucleohistone fibrils of interphase chromatin and metaphase chromosomes (Littau, Burdick, Allfrey & Mirsky 1965 and Mirsky, Burdick, Davidson & Littau 1968).

The primary structure of the histone molecules is very irregular as regards the distribution of the basic amino acids, and some molecules contain sequences of ten or more non-basic amino acid. (Phillips & Simson 1962, Johns 1964b and De Lange, Fambrough, Smith & Bonner 1969). It is possible that, owing to the spacing and sequence of basic residues within the F1 molecule, maximum electrostatic neutralization of DNA phosphates cannot be achieved. This would explain the striking fact that, although F1 has a greater number of basic residues per molecule than any other major histone fraction, it can be dissociated from nucleohistone at a lower NaCl concentration than any other fraction (Ohlenbusch, Olivera, Tuan & Davidson 1967). The weaker binding of lysyl groups than of

arginyl groups to native DNA (Leng & Felsenfeld 1966) could also contribute to the relative ease with which F1 can be dissociated. Nevertheless, although the binding of F1 to DNA is loose, the F1 molecule covers much more DNA per weight of protein than do the other histone fractions.

2. Interference of polyamines with nucleohistone formation (Paper 3).

Histones attach to DNA by electrostatic linkages between their basic residues, lysine, arginine, and histidine, and the phosphate groups of the DNA. The fact that high concentrations of salt (1 M NaCl) are able to abolish DNA-histone interaction (Papers 2 and 3) demonstrates its largely ionic nature. One might therefore expect polyamines to compete with histones for the phosphate groups.

DNA. Spermine, the polyamine which establishes the strongest bonds with DNA (Papers 1 and 2), was tested for its ability to compete with F1 and F3 for native DNA (Paper 3). Although spermine molecules are thought to bind principally within the small groove of the DNA helix (Liquori et al. 1967, Suwalsky et al. 1969), and histone molecules principally within the large groove (Olins 1969, Simpson 1970), the capacity of spermine and the two histones to compete for the phosphate groups of DNA was very nearly the same. Thus, although only about half of the DNA phosphate groups can be neutralized by the basic residues of histones (Klein & Szirmai 1963, Miura & Ohba 1967, Richards & Pardon 1970, Itzhaki 1970, 1971), spermine competes on equal terms with F1 and F3. This indicates that polyamines and histones compete for the same sites on the DNA molecule. It has been suggested that the free charge in the nucleohistone may be available to bind small molecules. However, if polyamines bind to these sites, an equivalent amount of histone must become loosened

and dissociate, since spermine competed with the histones on equal terms. This suggests the interesting possibility that polyamines may be concerned in the labilization of the histone-DNA complex in living organisms.

RNA. In RNA, where base pairing is realized to a much lesser degree, the heterocyclic bases are more readily available for combination with other molecules. To explain the higher affinity for RNA of spermine compared with F1, we considered a further possible mode of binding spermine to RNA. Observation of the RNA/spermine molar ratio (Paper 2) showed that an excess of spermine molecules binds to RNA and thus indicated the existence of additional binding sites. The excess basic groups of the spermine may be bonded to heterocyclic bases from which protons have become displaced (Huang & Felsenfeld 1960). On the other hand, spermine competes on about equal terms with F3 for RNA. This suggests that the additional binding sites can be utilized by F3 to almost the same extent as by spermine. Although differences in electrostatic attraction may contribute to the differential behaviour of the histones, differences in their structure ought to be of crucial importance for their attachment to nucleic acids.

3. Physical state of the chromatin.

The physical state of the chromatin in the interphase nucleus is the product of a complex set of interactions between DNA strands, histones, and other components of the nucleus. The histones act directly upon the DNA, causing charge neutralization, supercoiling, and perhaps cross-linking of DNA helices.

Wilkins, Zubay & Wilson (1959) were the first to suggest that in native deoxyribonucleoprotein the DNA diad helix is coiled again to form

what is now known as a superhelix or a supercoil. X-ray diffraction investigations on isolated nucleohistone have provided very strong evidence for a native supercoiled conformation of individual nucleohistone molecules (Pardon, Wilkins & Richards 1967). Supercoiling does not occur with DNA alone - it is entirely dependent on the histones. Fl does not, however, seem to be involved in the formation of supercoils (Bradbury 1969). It is possible that the hydrophobic loops of the histones not in close contact with the phosphates of the diad helix form hydrophobic bonds with corresponding loops further down the chain, thereby resulting in the formation of a supercoil. The macrostructure of the chromatin could then be built up from these coils by cross-linkage of the acidic amino acid side chains of the histones by cations such as Mg^{2+} , Ca^{2+} , putrescine, spermidine or spermine. The entire structure, being held together by hydrophobic and ionic bonds, would be very susceptible to small changes in ionic environment. Such changes may be part of the mechanism of condensation and subsequent diffusion of the chromatin during the cell cycle.

C. The role of polyamines and histones in gene expression

The histones are thought to play a part in the control of gene expression and differentiation by restricting the transcription of DNA. The evidence is that the template activity of DNA is decreased when it is combined with histones as in native chromatin or in reconstituted nucleohistone (Bonner & Huang 1963, Allfrey, Littau & Mirsky 1963, Barr & Butler 1963, Hindley 1963, Huang et al. 1964). Most and possibly all the DNA in the nucleus is complexed with histone as nucleohistone, in such a way that the DNA is clearly incapable of transcription. However, there seems to be no evidence at present that the histones need to be removed from DNA in vivo to enable it to be used as a template for RNA synthesis (Byvoet 1966, Hancock 1969). In the active regions of *Drosophila* salivary glands (Swift 1964) and in the active diffuse chromatin of interphase lymphocytes (Frenster 1965) there is an increase in the amount of acidic proteins and RNA, but never a significant decrease in the content of histones relative to DNA. It is only necessary to open up the structure of deoxyribonucleoprotein to obtain an increase in template activity (Sonnenberg & Zubay 1965). It now seems that the histones are non-specific gene repressors possessing an important structural function in maintaining the partially supercoiled structure of chromatin and thereby restricting transcription (Johns 1969).

Other molecules, which must have the necessary specificity to recognize a particular gene, may cause a derepression by breaking the very weak bonds of the supercoil and thus making the region available for transcription by RNA polymerase. Johns (1969) has suggested that the supercoil configuration will not permit the passage of a large molecule

like the DNA dependent RNA polymerase and that it therefore inhibits transcription. However, the non-supercoiling F1 also inhibits the template activity of DNA and therefore the presence or absence of supercoil cannot be the only factor involved.

There is good evidence that polyamines stimulate the synthesis of RNA under certain conditions (Krakow 1963, Fox & Weiss 1964, Abraham 1968, Barros & Giudice 1968, Petersen & Kröger 1970). It has been suggested that the polyamines act by combining with either DNA or RNA rather than with the RNA polymerase. Nevertheless, when explaining the stimulatory effect of polyamines on DNA transcription, their possible competition with histones has rarely been taken into account. Schwimmer (1968), however, suggested that putrescine and cadaverine might act by displacing the histones from the chromatin, since he had found that these diamines stimulated DNA synthesis if chromatin was used as primer. In addition to other possible modes of action, the polyamines may stimulate DNA replication and transcription by affecting the DNA-histone complex. The basic groups of the polyamines may effectively neutralize the DNA phosphates, with a consequent reduction of the strength of the bonds between the histones and DNA. This might be enough to open up the structure of deoxyribonucleoprotein, thus causing an increase in template activity. It is not likely that the stimulatory effect of polyamines on DNA and RNA synthesis can be explained as an effect on supercoil conformation, since they would tend to stabilize rather than destabilize the structure, by cross-linking acidic amino acid side chains of the histones.

PART II

POLYAMIDES IN GROWTH AND DEVELOPMENT

A. Embryonic and postembryonic development (Paper 4)

Polyamine synthesis and accumulation are apparent early in embryonic growth and development. Elevated activities of S-adenosyl-L-methionine decarboxylase (Russell 1970, Russell & Lombardini 1971) and ornithine decarboxylase (Russell & Snyder 1968, Snyder & Russell 1970, Snyder, Kreuz, Medina & Russell 1970) and the subsequent accumulation of polyamines (Raina 1963, Caldarera, Barbiroli & Moruzzi 1965, Caldarera & Moruzzi 1970, Russell & Lombardini 1971) have been reported in developing chick embryos. Similar changes have been observed in embryonic and foetal rats (Snyder & Russell 1970, Snyder, Kreuz, Medina & Russell 1970, Russell 1970) and during the development of the South African clawed toad, *Xenopus laevis* (Russell, Snyder & Medina 1969, Snyder, Kreuz, Medina & Russell 1970, Russell 1970, 1971). The increases in enzyme activities in the amphibian, chick and rat embryos, were apparent in all tissues examined. The changes in polyamines during embryonic growth and development tended to parallel changes in DNA and RNA. Of particular interest was the observation that the synthesis of putrescine and spermidine in embryos of *Xenopus laevis* was correlated with the onset of ribosomal RNA synthesis and the formation of a viable nucleolus in the embryonic cell (Russell, Snyder & Medina 1969, Russell 1970, 1971). It thus appears that stimulation of polyamine synthesis is a universal feature of embryonic growth and development. By investigating the possible occurrence of and variations in polyamines during the developmental cycle of the blowfly, *Calliphora erythrocephala*, it was hoped to elucidate the interaction between nucleic acids, basic proteins, and polyamines during periods of growth, histolysis, histogenesis and differentiation.

All developmental stages of the blowfly, *Calliphora erythrocephala*, contained considerable amounts of polyamines, spermidine being present at a higher concentration than putrescine and spermine. Spermine constituted only a minor proportion of the polyamines.

During the development of the blowfly, growth is restricted to the early larval period. Curiously, larval growth occurs by an enormous enlargement of the cells, which is not restricted to the cytoplasm but also involves the nucleus. A very high degree of polyploidy can thereby be attained (for references see Agrell 1964). Newly hatched and vigorously feeding larvae exhibited particularly high polyamine concentrations, in accordance with previous findings on another endopterygote insect, *Drosophila melanogaster* (Herbst & Dion 1970, Dion & Herbst 1970). The concentration of spermidine in fertilized eggs and early embryonic stages of developing *Drosophila* was 2.5 times the level of spermidine in subsequent developmental stages, and the most rapid increase in the content of spermidine per insect coincided exactly with attainment of maximal rates of RNA and protein synthesis during larval growth. A report by Church & Robertson (1966) concerning times of maximal synthesis of DNA-like RNA and ribosomal RNA during larval development corroborates the contention of Herbst & Dion (1970) of a close association between spermidine and biochemical growth parameters.

Fat bodies from 4-day-old larvae, which constitute nearly half of the larval weight, are rich in ribosomes, whereas those from 7-day-old larvae contain very few ribosomes (Price 1969). It was suggested that the ribosomes were broken down as the larvae aged, but it was also found that the amount of RNA was still the same. Therefore RNA from the ribosomes was considered to provide a store for subsequent needs. Polyamines

have been shown to cause significant inhibition of ribonuclease action (Herbst & Doctor 1959, Amos & Kearns 1963, Gabbay & Shimshak 1968), and to increase RNA accumulation (Goldstein 1965, Raina & Jänne 1968b). I therefore consider it likely that spermidine, which exhibits a peak value at day 7 (Paper 4), protects RNA molecules that are no longer associated with protein in ribosome formation from ribonuclease action.

The concentrations of both putrescine and spermidine increased rapidly during the period of histolysis, indicating that appropriate precursors were set free and utilized for de novo synthesis of polyamines during the breakdown of larval tissue. This view is strengthened by the observation that the principal synthesis of polyamines during the period of histolysis occurred in the abdomen, the body part which exhibits the most predominant histolysis during this period. After histolysis the amounts of polyamines gradually decreased in the abdomen, while they maintained high and rather constant levels in the head and thorax during histogenesis. This might imply that the polyamines are involved in the growth of the flight muscles, possibly as stimulators of RNA and protein synthesis.

The main bulk of the polyamines cannot be associated with DNA, since the DNA concentration in the blowfly is too low (Agrell 1964). However, the changes in the amount of spermine closely parallel those in DNA and, as the two molecules have been shown to establish strong electrostatic bonds in model experiments (Paper 2), this condition may also appear *in vivo*.

Polyamines, as well as basic proteins (Agrell & Lindh 1966) in excess of DNA, seem to be RNA-linked. One RNA fraction (high molecular weight RNA of the cytoplasm and nuclear sap plus the total low molecular

weight RNA; Georgiev, Samarina, Lerman, Smirnov & Severtzov 1963) varies in quantity in almost the same manner as the basic proteins (Agrell & Lindh 1966). The other RNA fraction (RNA of nucleoli and chromosomes; Georgiev et al. 1963) varies in almost the same manner as the polyamines. Whether the polyamines actually are combined with "nuclear" RNA, or whether the similarity in their patterns of variation is coincidental, cannot be settled at present. However, many observations lend support to the concept of polyamine-"nuclear" RNA interaction (Dion & Herbst 1967, Gfeller & Russell 1970).

The polyamine changes which occur during development can be related to rapid growth processes and are indicative of the active participation of these compounds, especially spermidine, in the regulated synthesis of nucleic acid and protein.

B. Cell growth and synchronous division (Paper 5)

The presence of putrescine, spermidine and spermine in *Tetrahymena pyriformis*, amiconucleate strain GL, has been established by Weller, Raina & Johnstone (1968) and Arlock, Heby & Holm (1969).

The quantity of polyamines in heat-treated *Tetrahymena* cells was analysed during cell growth (heat treatment plus the subsequent recovery period) and cell division. The intracellular amount of putrescine exceeded that of spermidine by a factor of more than two. Spermine was present, but only in very small amounts. There was a 2-3 fold increase in the endogenous level of putrescine and spermidine during cellular growth. Correspondingly, the amount of protein and of RNA is 2-3 times greater in heat-treated cells than in exponentially growing cells (Christensson 1959, 1962).

During cell growth two maxima appeared in the putrescine concentration. The first significant increase occurred between the 6th and 7th heat shocks, and the second during the recovery period just before the 1st synchronous division. In view of the variations in ^3H -putrescine uptake from the medium (Holm & Emanuelsson 1971) and the pattern of endogenous accumulation of putrescine (Paper 5), it seems that both the synthesis and the uptake of putrescine is disturbed by the heat treatment and that putrescine can accumulate only under optimal temperature conditions. The explanation of the observed peak value in the putrescine concentration between the last two heat shocks may be that this is the first occasion when the cell population is synchronous enough to enable the demonstration of a biochemical change related to a certain phase of the cell cycle. In addition to the increased accumulation of putrescine

during the recovery period, there is a concomitant increase in the synthesis of mRNA, sRNA and rRNA, and of protein (Christensson 1968). A comparable parallelism is observed between the 1st and 2nd divisions. An elevation of the putrescine level may be assumed to increase the template activity in the heat-treated cells and thus contribute to growth and division.

It is still not proven that putrescine actually is synthesized in *Tetrahymena* cells, although it would be surprising if it were not. In addition to endogenous synthesis of polyamines, the *Tetrahymena* cells may utilize exogenous sources of putrescine, spermidine and spermine. The culture medium contains these amines in concentrations constituting 1/5th to 1/8th of the intracellular concentrations. In accordance with our quantitative data, it was found that ^3H -putrescine, added to the culture medium, accumulated in the cells only between the heat shocks (Holm & Emanuelsson 1971).

C. Regenerative growth of the liver (Paper 6)

The hepatocytes of the adult mouse do not normally divide, but they can readily be provoked to active proliferation by partial hepatectomy, an operation that is well tolerated and has a low mortality. Since the two main lobes comprise approximately 70% of the total liver weight, a reproducible growth stimulus can be delivered by their removal. The growth process that is induced is not regeneration in the strictest sense, since the resected lobes are not reformed. Rather, it is a compensatory hyperplasia of the two residual lobes.

Within a few hours after resection, a number of morphological and biochemical changes occur in the liver remnant, and around 33 hours postoperatively there is a burst of DNA synthesis (Bade, Sadnik, Pilgrim & Maurer 1966), followed in 10-20 hours by mitosis (Paper 6). DNA synthesis is the earliest event that shows with certainty that cellular proliferation has been induced. This synthetic activity gradually tapers off during the ensuing two weeks as the liver regains its original mass. Only the hepatocytes respond in the initial stages, whereas the other cell types lag behind by about a day.

Increased polyamine concentrations in rat liver after partial hepatectomy have been reported by several laboratories (Dykstra & Herbst 1965, Raina, Jänne & Siimes 1966, Jänne 1967, Russell, Medina & Snyder 1970, Raina & Jänne 1970, Raina, Jänne, Hannonen & Hölttä 1970, Russell & Lombardini 1971). It has been suggested that most of the tissue polyamines are associated with RNA. The time course of biochemical events in regenerating mouse liver is very different from that in rat liver; both DNA and RNA synthesis occur much later. The question thus arose

as to whether polyamine accumulation would show a similar correlation to RNA accumulation in the mouse liver after a corresponding resection. We therefore undertook a study of polyamine variations in regenerating mouse liver and their relation to growth, mitosis, and nucleic acids.

The first change noted after partial hepatectomy was in the putrescine concentration, which reached 187% of the control level within 13 hours. The spermidine concentration reached a peak value of 146% of the control level at 70 hours. In contrast to the increases in the putrescine and spermidine concentrations, that of spermine decreased to a minimum of 71% of the control level at 70 hours postoperatively. The polyamine changes which we observed in regenerating mouse liver are quite comparable with the results of Russell & McVicker (1971).

The increased accumulation of putrescine in the regenerating liver is obviously due to the elevated ornithine decarboxylase activity (Russell & Snyder 1968, Jänne & Raina 1968, Fausto 1969, Snyder & Russell 1970, Snyder, Kreuz, Medina & Russell 1970, Schrock, Oakman & Bucher 1970, Russell & McVicker 1971). As putrescine has been shown greatly to stimulate the S-adenosyl-L-methionine decarboxylase system (Pegg & Williams-Ashman 1968b), the elevated putrescine pool may be the main cause of the increased synthesis and accumulation of spermidine. Indeed, the S-adenosyl-L-methionine decarboxylase activity has been shown to be enhanced after partial hepatectomy (Russell 1970, Russell & Lombardini 1971, Russell & McVicker 1971).

The pattern of spermidine accumulation resembled the RNA increment but there was not a general 1:1 relationship during the 140 hours of the experimental period. The maximal spermidine concentration was obtained about 20 hours before the maximal RNA concentration. Russell &

Lombardini (1971) have reported that the spermidine concentration remains elevated for over 2 weeks in rat liver, whereas the enhanced RNA concentration returns to a basal value within 8 days.

During the regeneration there was a marked decrease in the spermine concentration, but only a slight decrease in the DNA concentration. The decreased spermine concentration may indicate a degradation of spermine to spermidine, a metabolic pathway evident from observations on regenerating rat liver (Siimes 1967). As the spermine concentration decreases, the DNA may progressively become metabolically more active, due, for example, to labilization of its double-stranded structure. A higher template activity for RNA synthesis has been observed in regenerating liver chromatin, but no difference is detectable in the ratio of histone to DNA, compared to that of normal liver chromatin (Bannai & Terayama 1969). However, the interactions between DNA and its associated proteins may be expected to change at the time of gene activation, and the net increase in polyamines, accounted for by spermidine, may partially displace histones from the chromatin, thereby increasing its template activity. This explanation is supported by the observation that putrescine and spermidine stimulate, whereas spermine inhibits, RNA synthesis in rat liver nucleolar preparations (Raina & Jänne 1970). Putrescine also stimulates DNA synthesis if chromatin or nucleohistone are used as primers (Schwimmer 1968).

2. Tumour growth (Paper 7).

2.1. Changes in the tumour. The growth of the Ehrlich ascites tumour is characterized by a rapid initial phase, followed by a deceleration in growth rate. It seemed probable that the differences between early and late ascites tumour cells would be reflected in their intracellular polyamine concentrations, and we therefore analysed representative stages of tumour growth. Available information about biogenesis and accumulation of polyamines in Ehrlich ascites carcinoma cells (Benedict, Keeler-Ross & Ling 1967, Oliva & Jaffe 1967) and other rapidly proliferating tumours (Kowalski & Taylor 1966, Reiss & Kay 1967, 1968, Russell & Snyder 1968, Snyder & Russell 1970, Snyder, Krey, Medina & Russell 1970, Krummer, Barrett & Terrano 1970, Russell & Levy 1971) was restricted to samples from tumours whose phase of growth was not well defined. However, in the present investigation the polyamine concentrations in Ehrlich ascites tumour cells were determined at known intervals after inoculation of the tumour.

The putrescine concentration was markedly elevated in the latter phases of rapid multiplication, but declined considerably with increasing tumour mass. The patterns of change in the intracellular spermidine and spermine concentrations were similar to each other. Initially, they remained rather constant but, as the tumour entered the stationary growth phase, there was a temporary decrease in concentration, followed by a gradual increase. The spermidine concentration in the tumour cells was notably high and more than twice that of spermine during the entire growth period. The endogenous levels of polyamines in a tumour induced in mice by subcutaneous inoculation of 13210 ascites tumour cells (Russell &

Levy 1971), closely resemble the values we obtained for the Ehrlich ascites tumour.

A dramatic elevation in the activities of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase, occurs early in the sequence of event leading to proliferation (Russell & Levy 1971). In preliminary studies of ornithine decarboxylase activities in Ehrlich ascites carcinoma cells, we found that the activity of this enzyme was markedly elevated in the phases of most rapid multiplication, an observation that explains the concomitant accumulation of putrescine. The data obtained lend further support to the hypothesis that there is some connection between rapid growth and an elevated putrescine concentration. Our results demonstrate a definite quantitative pattern in the spermidine-N/nucleic acid-P and spermine-N/nucleic acid-P ratios during tumour growth. It has been proposed that the condensation of chromatin results from an interaction between positively charged substances like polyamines and the negatively charged DNA strands of the chromosomes (Anderson & Norris 1960, Rao 1969). The changes in the polyamine-N/nucleic acid-P ratios may therefore reflect the accumulation of pro-phases that peaks on the 11th day of tumour growth.

2. Studies on the liver of tumour-bearing animals. Little information is available about the possible influence of tumour growth elsewhere in the body upon the concentration of liver polyamines. It was therefore decided to investigate whether the concentrations of polyamines in mouse liver were influenced by Ehrlich ascites tumour growth, as shown for the livers of rats bearing solid Rd/3 sarcomas (Neish & Key 1967).

The results clearly demonstrated that the transplanted ascites tumour greatly influenced the spermidine concentration in the liver. After a precipitous drop, it progressively increased to a value considerably higher than the initial value. However, only minor changes were seen in the putrescine and spermine concentrations. A comparable accumulation of spermidine has previously been observed in the livers of rats bearing solid Pd/3 sarcomas (Neish & Key 1967) and in the livers of pregnant rats (Neish & Zay 1968). There was an increase in the nuclear diameter of the hepatic parenchymal cells of our tumour-bearing mice, which agrees with previous findings from other tumour-bearing animals (Sieburgs, Parets, Perez & Boudreau 1965, Rosene 1966, Hoch-Ligeti, Stutzman, Brown, Grantham & Arvin 1969). Since polyamines stabilize RNA, and the nuclear RNA content of liver cells is significantly increased in cancer patients (Hoch-Ligeti et al. 1969), the progressive increase in spermidine concentration possibly serves to stabilize newly synthesized RNA molecules in the livers of tumour-bearing mice.

SUMMARY AND CONCLUSIONS

The quantitative relations between polyamines and nucleic acids were analysed in model experiments (PART I) and in various growth systems (PART II).

1. The affinity between various polyamines and nucleic acids was studied systematically in terms of their mutual precipitability. On the basis of the results tentative molecular models were proposed for the precipitated polyamine-nucleic acid complexes.
2. Spermine interfered with nucleohistone formation in model experiments and it is suggested that polyamines might assist in the labilization of the histone-DNA complex in living organisms thereby increasing the template activity.
3. During the postembryonic development of the blowfly, *Calliphora erythrocephala*, there was a close association between polyamine content and biochemical growth parameters. A possible interaction with "nuclear" RNA was indicated.
4. Heat-treatment, which affects the rate of RNA and protein synthesis in *Tetrahymena* cells, also affected the intracellular concentration of polyamines.

5. Partial hepatectomy caused an early accumulation of putrescine in the mouse liver and there was a close correlation between the concentration of spermidine and the accumulation of RNA during the regenerative process.
6. In Ehrlich mouse ascites carcinoma cells, the putrescine concentration was markedly elevated in the phases of most rapid multiplication. The high concentrations of spermidine in the tumour cells may reflect their rapid growth.
7. The transplanted ascites tumour greatly influenced the spermidine concentration in the liver. It did not, however, markedly alter the DNA and RNA concentrations.
8. Increasing support has been lent to the view that polyamines perform an important role in growth processes. Experimental evidence has been obtained to suggest that cellular polyamines are physiologically associated with nucleic acids. It may be speculated that changes in the concentrations of polyamines affect the rate of transcription, and the experimental evidence obtained by studying the effect of polyamines on nucleic acids in model experiments appears to offer support for this hypothesis. It also seems very possible that, in addition to stimulating the rate of RNA synthesis, the polyamines may decrease the rate of degradation of newly synthesized RNA molecules and thereby increase the accumulation of RNA.

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